Spinnability of collagen as a biomimetic material: a review

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Abstract

In this review, an attempt was made to summarize some of the recent developments in the spinnability of purified collagen. Due to the excellent biological properties of this biopolymer, it is often chosen among other biomimetic materials for processing into fibrous assemblies. During the last two decades, the challenges associated with regenerated collagen fibers comprising inability to achieve sufficient tensile strength, reproducibility and failure to replicate the internal fibrillar structure, which are due to the lost properties from hierarchical structure consistent with collagen in native tissues, have been considered using the common spinning and the modification methods. Among the common spinning methods, dry spinning and wet spinning result in well-defined fibrous blocks with relatively high fiber diameters and alignment, while the ability of the electrospinning to fabricate custom-built nanofibers from collagen-based composites may be the main reason that made it the most applied method to mimic the structure of the collagen in native tissues. In this review, the modification and spinning methods, used for forming collagen fibers, were summarized and their strategy to achieve the modified and reinforced collagen fiber was studied.

Key words: Collagen, Chain entanglements, Fiber spinning, Cross-linking, Blends, grating polymerization, non-covalent conjugation, Nanofiller

Introduction

One of the most important and essential aspects in material development is the selection of a starting material; usually one that is highly available and to some extent achieves added value when is processed [1-5]. Collagen is one of the most abundant biopolymers within biomimetic materials. It is available in the extracellular matrices of many connective tissues of mammals, comprising about 25-35% of the whole-body protein contents [3, 6-8]. Collagen is typically found in fibrous tissues such as tendons, skin, and ligaments, which comprise about one half of total body. It is also abundant in cartilages, bones, corneas, gut and blood vessels [8]. Thus far, 29 types of collagen have been recognized and categorized. Among them, type I forms over 90% of the collagen of the body, which is commonly found in tendons, skin, bones, ligatures, vascular, and organs; even though these statistics can differ with age and injuries [9].

It is widely agreed that collagen provides mechanical stability, strength, and elasticity to native tissue and it is the main structural material in biology [10-12]. As shown in Figure 1[13], a collagen fibril is fully constructed from the biochemical details of the amino acid sequence and nanoscale chain arrangements that benefit from supplementary chain interactions showed in Figure 2 [14]. According to Gautieri et al. [10], the fully hydrated collagen fibrils exhibit Young's modulus of about 300 MPa and 1.2 GPa for lower and larger deformation strain of 10%, respectively. The dehydrated collagen fibrils represent a considerably increased Young's modulus of about 1.8 to 2.25 GPa due to tighter molecular packing. From their experimental data and numerical analysis, they suggested that the high mechanical properties of collagen in native tissues are due to the hierarchical structure of the collagen molecular chains in the nanoscale, where the deformation mechanism comprises the straightening of twisted triple-helical collagen chains and then axial stretching followed by chain coiling.

This claim has been studied by some other research groups, confirming the stability and the strength of collagen that are induced by its hierarchical structure in native tissues [15-17]. By contrast, it is widely accepted that uncoiled collagen chains (α- chains) in the primary and secondary structures cannot provide the required mechanical functionality for native tissues. In fact, uncoiled collagen chains, the product of the purification and isolation process, are

used as purified collagen and typically prepared by alkaline or acid hydrolysis of animal skin and bones [18].

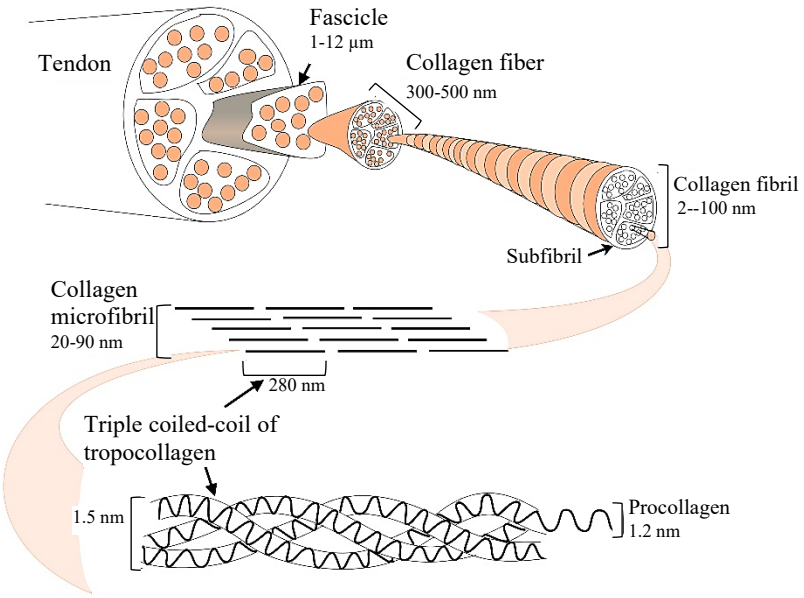

Figure 1 Hierarchical structures in fibrillar collagen show a characteristic periodic structure forming various hierarchical orders of association with their distinctive features. Reprinted from Elsevier, Scarr G., Simple geometry in complex organisms, J. Bodyw Mov Ther 2010; 14:424-44, with permission from Elsevier [13].

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Despite the fact that collagen is one of the most abundant proteins, purified collagen loses some properties induced from the hierarchical structure in native tissue such as mechanical strength, however, the unique physiochemical properties from α- chains are still present thereafter. More specifically, they can enhance the carrier systems of other materials when they are processed as composite fibers, sponge or film. This is due to the high reactivity of this biopolymer with other materials such as drugs, cells, ions, (bio)polymers and (nano)fillers [19-21].

Over the past decade, to mimic the structure of collagen fibrils in native tissues when the properties associated with increased surface area to volume ratio are tailored, fibrous assemblies received a great deal of attention within clinical treatments, such as in drug delivery systems, wound and burn dressings, heart valves, nerve regeneration, ocular surfaces and vascular grafts [8]. Hence, in this review, we are to study recent successful collagen-based fiber spinning including the effect of modification methods, process conditions, and the source of the collagen in relation with the spinnability of this biopolymer.

1.1 Effect of modification methods on spinnability of purified Collagen chains

Since purified collagen represents poor mechanical strength, low dimensional stability, reduced elasticity, and also high degree of hydration and eventually rapid degradation rate, its polymeric chains are subjected to modification methods, mainly focusing on reducing the super hydrophilicity of the purified collagen chains[22]. To the best of our knowledge, apart from the severity of the above-mentioned drawbacks, the purified collagen chains cannot be exploited free from modification as either pre– or post–treatments. These methods are typically to stabilize collagen chains into an engineered biopolymer [23, 24]. So far, the spinnability of this biopolymer has been explored by using four possible approaches as modification methods: cross-linking, blending, grafting and conjugating.

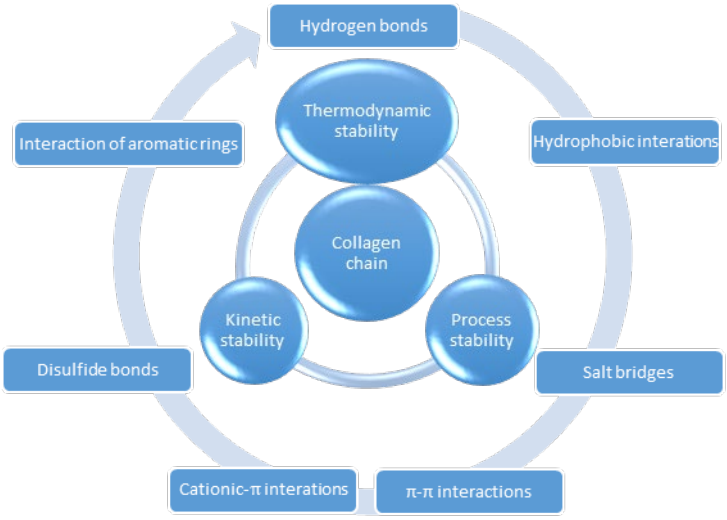


Figure 2 Summary of inter– and intra–molecular bonds available in hierarchical arrangements affecting the process, kinetic and thermodynamic stability of the collagen chains as a protein [14].

1.1.1 Crosslinking modification approach

Crosslinking is the process of chemically/physically joining two or more molecules by a covalent/non-covalent bond. This approach involves attaching or cleaving chemical groups to alter the solubility or other properties of the purified collagen chains.[25]

Many studies have been dedicated to the crosslinking modifications, resulting in a fibrous product with a higher collagen chain content. However, non-uniformity in cross-linking is more likely to occur since this method is mostly implemented as post-treatment onto the surface of collagen fibers. Meanwhile, the fact that the unavoidable addition of unreacted toxic agents may exist in the final product, can make this approach problematic and compromise specific end-uses [26].

For instance, Aldehydes such as glutaraldehyde (GTA) and formaldehyde (FA) are bi-functional reagents, which are typically used to chemically modify polypeptides and polymers for various applications due to its high reactivity, availability and low cost [27-30]. They covalently bond to amino acids but can also bind to other similar chains, increasing the non-uniformity in cross-linking. The direct usage of high concentrations of aldehydes may prove challenging for fibers to be used in hygiene and medical products. Also, due to low level of cross-linking of the aldehydes in low concentrations, the performance of the fibers from poly-peptides are likely to be reduced by low uniformity of cross-linked amino acid chains [27]. To eliminate the effect of toxic cross-linking agents in collagen-based fibers, cross-linking with sugars (e.g. Genipin) for pharmaceutical applications has been also used, which can also boost the water resistance and mechanical strength [31, 32]. This non-permanent cross linking is regarded as the Maillard reaction, which is a reversible chemical attachment between sugars and amino acids; this reaction only makes physical changes in the polypeptides [33].

The zero-length cross-linker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in combination with *N*-hydroxysuccinimide (NHS) are also the other common crosslinking agents that make the collagen stabilized by catalyzing covalent bonds between amino and carboxyl groups [34, 35]. Again, other components containing carboxyl groups, such as glycosaminoglycans, can also be cross-linked.

In addition, Transglutaminase (TGase) is a highly specific enzyme catalyzing collagen cross-linking between intra- and inter-chain glutamine and lysine peptide residues in collagen structures with the release of ammonia, but this method only targets specific amino acids

[36]. To examine the functionality of the cross-linked collagen-based fibers, Huang et al. [31] used four different cross-linkers; GTA, Genipin, EDC, and NHS. They observed varied physical properties and biological behavior on fibers from different cross-linkers: e.g., the fiber morphology of fibers vanished with GTA; Genipin preserved the fibers architecture only for a short time period and crosslinking with an EDC-NHS combination showed better results in preserving the fiber morphology after process optimization.
There are also some other post-treatment (e.g. UV radiation, gamma radiation, dehydro-thermal treatment) identified as physical treatment for collagen cross-linking [37]. According to Tonndorf et al. [26], it was evaluated whether riboflavin-induced photo-crosslinking could be used as a non-toxic alternative to glutaraldehyde (GTA)-crosslinking for the preparation of wet spun collagen filaments. They successfully concluded that the combination of riboflavin and UV light leads to cross-linked collagen filaments as GTA does. Furthermore, riboflavin-crosslinked filaments exhibited a higher cytocompatibility for human mesenchymal stem cells compared to GTA-crosslinked filaments.
Interestingly, to enhance the mechanical strength of the collagen fibers, there are some efforts using epoxy compounds as cross-linkers to be processed along the collagen chains as a polymer matrix or coat the superficial surface of the processed collagen chains [38]. Cross-linking with epoxy compounds such as ethylene glycol diglycidyl ether, diglycerol triglycidyl ether and allyl glycidyl ether, has been claimed to maintain good biocompatibility while enhancing mechanical properties and water resistance [38, 39]. However, the reaction of collagen chains with epoxy compound through the reduction of the amine groups (NH_2) as a function of time, temperature and pH, is the matter of fact that increase the processing time [40, 41]. This claim was examined by Stoessel et al. [42] when they used a complex blending system including a set of polymers and cross-linking agent (ethylene glycol diglycidyl ether, EGDE) to achieve cross-linked wet-spun fibers, as shown in Figure 3. However, they recommended that to achieve more stable fibers in unstable humidity, double-cross linking is required as post-treatment; water-resistant fibers cross linked by epoxy compounds followed by post treatment of FA. Fukai et al. [28] also suggested that double crosslinking using gamma-Irradiation and treatment with GTA improve the stability of the collagen fibers.
Although using epoxy compounds have received a great deal of attention from conventional fiber spinning methods such as wet spinning and gel spinning [27, 28, 42], to the best of our knowledge, fewer studies have been dealt with this cross-linking approach processing via electrospinning method.
In general, any chemical/physical post treatment may produce a low degree of cross-linking, as the reaction is likely to only happen on the surface of the fibers; the resorption rate, strength and biocompatibility of collagen-based fibers are greatly influenced by the method and extent of cross-linking [25]. Also, from our understanding, the cross-linking process when is used as post-treatment on spun fibers, the morphology and hydrophilicity of spun fibers are difficult to be optimized simultaneously as a function of exposure time, temperature, and concentration.

1.1.2 Blending modification approach

Researchers have also focused on blending systems of electrically and structurally compatible polymers to enhance the physiochemical properties of collagen fibers such as mechanical and biological. For instance, Sionkowska et al. [43] characterized the intermolecular interactions of collagen and chitosan blends as Polycations. They found that the hydrogen bond forces between the collagen and chitosan resulting in collagen-chitosan blends being miscible and spinnable simultaneously. This example has been studied by several research groups using different spinning methods; for the first time Hirano [24] et al. investigated the wet spun

collagen-chitosan and Chen et al. [21] also examined the spinnability of collagen-chitosan blends through electrospinning to enhance the mechanical properties of collagen chains.

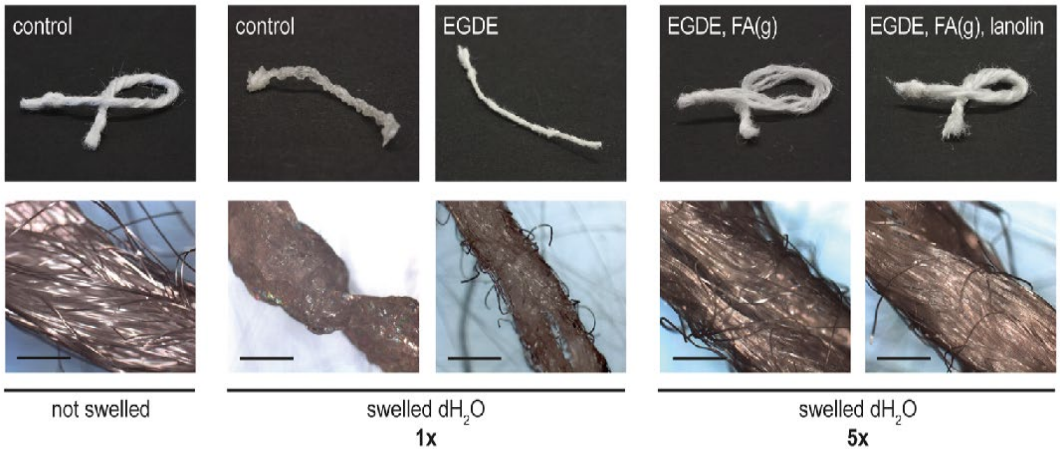


Figure 3 Images of collagen-based yarns representing the requirement of double crosslinking through epoxy compounds followed by aldehydes as post treatment to maintain the morphology of the fibers in high humidity conditions. Reprinted with permission from (Ref. no. [42]). Copyright (2015) American Chemical Society.

From their results, the optimum biological and mechanical properties were reached in lower collagen contents when the ratio of collagen-chitosan was 1:4. From the above-mentioned examples, a blending system can be useful for mechanical reinforcement; however, reduction in collagen content is unavoidable due to the presence of other polymers [44], furthermore according to recently published papers, the resultant fibers are still required to be cross-linked [45, 46].

Even though, this modification method is not as simple as it seems; phase segregations and viscosity changes are simple examples of incompatible (co)polymeric fluids. Phase segregation is a common challenge that needs to be considered in material selection, when polyelectrolytes can form a network of electrostatic attractions and repulsions before and during the process (fiber spinning) [47, 48]. The formation of electrostatically charged network is highly dependent on the characteristics of both electrolytes, such as charge density, chain length (molecular weight), ionic strength, and concentration of polymer solution. Many investigators have reported that increasing concentrations of polyelectrolytes in solutions leads to formation of larger network [49]. This critical issue has significantly limited the use of collagen-based materials processing along with structurally and dynamically rich polyanions in blends [47, 50].

1.1.3 Grafting modification approach

The other identified modification method for biopolymers and more specifically, for collagen, is grafting polymerization. In the past, the effects of various parameters in grafting modification onto biopolymers have been investigated to form corresponding radical on the chains [51, 52]. Those investigations were conducted radicals by applying varied kind of vinyl monomers such as butyl acrylate , ethyl acrylate, methyl acrylate, acrylonitrile, methyl methacrylate, and methyl methacrylate-co-ethyl acrylate with a varied amount of initiators [53-55]. The main objective of most investigations in this field have been also focused on reducing the hydrophilic behavior and possessing the collagen, however due to structurally changed collagen chains into a branch structure, the collagen graft copolymer may receive new properties from the new molecular structure of branching and the branches themselves vary from their origin. Increased viscosity and density are one of the most important factors

that needs to be considered for fiber spinning to prevent non-uniform fiber formation [56, 57]. For example, CN0214594 Chinese Patent entitled "collagen composite fiber and its production method" revealed a method of producing biocompatible collagen/polyvinyl alcohol (PVA) fibers from purified collagen modified by grafting with olefin monomers and mixed with PVA to form a spinning solution, and processed through a complex procedure using wet spinning, stretching and further post-treatment (acetalization). This example signifies the grafting methods combined with blending systems to form spinning liquid with certain density and viscosity.

Little attention has been given to development of collagen-based fibers which have been structurally modified by graft polymerization, existing research to align collagen chains and produce collagen fibers is scarce. This is likely to be due to the fact that the grafting of vinyl monomers leads to modification of structural, rheological and morphological properties. However, in our previous work, grafting polymerization of methyl methacrylate-*co*-ethyl acrylate was applied to modify the surface of collagen chains. We realized that the branched copolymer on the surface of collagen significantly influenced the initial viscosity. Since chain entanglement is crucial for fiber formation during electrospinning, the dependency of entanglement concentration on branch densities possessing the same viscosity was investigated; in which the mean fiber diameters of all considered samples remained broadly constant. We found that increasing the number of branching onto collagen chains significantly increased the stability of the collagen-based fibers under high humidity conditions and the long chain branches can provide a higher chain entanglement density leading to the more fiber uniformity [57].

However, there are some interesting studies considering the collagen properties that are covalently bonded on a pre-processed substrate. A pre-processed substrate can be in the form of fibers, fabrics, films of polymers, and composites. Jou et al. [58] reported Fibers of poly(ethylene terephthalate) (PET) were grafted with acrylic acid. The resulting fibers were further grafted with chitosan and collagen by means of esterification. Their results indicated that growing branches of chitin-collagen on PET fibers improved the multi-functionality of the composite fibers; whereas Yuan et al. [59] implemented almost the same procedure focusing on surface modification of PCL substrates using collagen covalently immobilized by poly (meth acrylic acid) via surface-initiated atom transfer radical polymerization.

1.1.4 Conjugation modification approach

The field of covalently conjugation and its potentials have already been proved by many research groups for the delivery of proteins and drugs. To enhance the miscibility of peptides with specific polymers or other proteins, this approach has been used, which is normally mediated by NHS and EDC in a controlled reaction time and pH [60].

As an example, a successful attempt to prepare a biosynthetic collagen-based copolymer has been reported by Gentile et al [61]. They electrospun a solution of conjugated PCL/Collagen where the conjugation points were evidenced by the presence of C–N and N–C=O bonds, the reduced fiber diameter was observed in compare with normal PCL/Collagen blends. In their study, this approach was to improve the miscibility of these two useful biopolymers benefitting from good mechanical strength and biological properties simultaneously. They demonstrated that PCL has attractive mechanical and physicochemical properties and collagen improves cellular adhesion and growth. However, from their observation on potential collagen release in water, post treatment of cross-linking may be required for some applications applying this modification method.

On the other hand, recently some interesting studies have been also reported focusing on increasing the stability of collagen chains in high humidity conditions by conjugating with active nanofillers such as Graphene oxide (GO). The covalent conjugation of GO to collagen

nanocomposite have been evidenced by significantly reduced oxygen and carbon dioxide permeation on GO plane and edges [56, 62] and the increased tensile strength without cytotoxicity has been reported in low concentrations of GO in fibers [62, 63].
However, according to Panzavolta et al. [62], the size of the nanofillers when spun into nanofibers can be problematic causing non uniformity and increased fiber diameter ; the size of GO sheets is comparable to the size of e.g. the nanofibers (~ 200 nm, Figure 4), even though this GO decoration can be beneficial for applications that require highly active functional groups of GO on the surface of the fibers [56]. Furthermore, the high chemical affinity of these two materials hinders the denaturation of collagen chains and origins a defect free mixing for GO–collagen composites. This claim were also proved in our previous study[56] benefitting from covalent and non-covalent conjugation of the GO–collagen nano composite fibers, however we observed that the water stability of GO–collagen composite fibers is dependent on the temperature when polymer chains are unpacked due to increase molecular mobility that influenced by increased Temperature above glass transition temperature (Tg) ; by increasing the temperature above 50ºC, the collagen release may be accelerated in water (high humidity conditions).

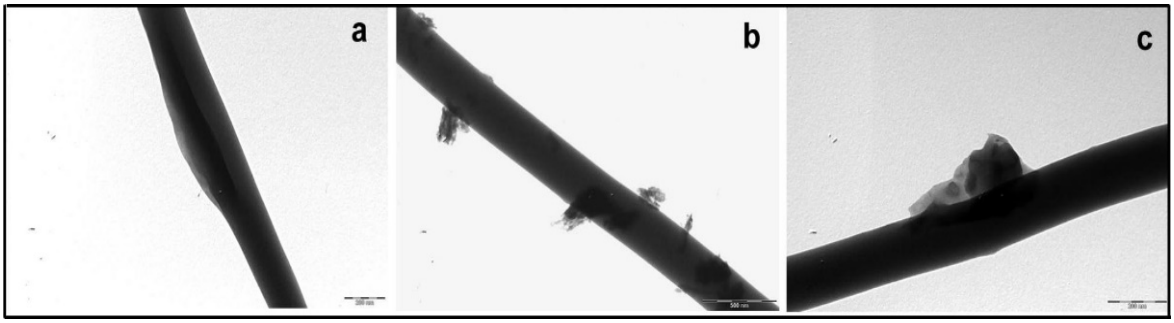


Figure 4 TEM images of electrospun mat showing GO sheets decorated (b) on the surface or (a and c) partially embedded into collagen-based fibers. Scale bar: 200 nm (a and c); 500 nm (b). Reprinted from (Ref. no. [62]) Copyright (2014) with permission from Elsevier.

1.2 Effect of fiber spinning methods on the processability of collagen chains

In general, a spinning method is chosen by considering the properties of the material(s) to be spun into fibers. Due to the low denaturation temperature of collagen, it is problematic to utilize melt-spinning method. Hence, collagen is typically processed via solution-based spinning methods; two key strategies may be considered:

(i) Conventional fiber-spinning through methods comprising dry-spinning [64, 65], gel-spinning [28, 66], and wet-spinning [27, 67-69], which are typically processed by post-drawing to achieve aligned fibers with high throughput due to process conditions;

(ii) Direct fiber formation into micro- or nano-sized fibers by using electrospinning, which is tailored due to the properties in relation with the increased surface to volume ratio of accumulated fibers.

To process the collagen chains via conventional fiber spinning methods, they must be converted into a fluid phase, mechanically forced through a spinneret that creates collagen fibers, and specifically in wet spinning, the collagen fibers are extruded into a non-solvent and precipitation or coagulation occurs [70]. To the best of our knowledge, protein-based fibers have been prepared mostly by wet spinning among conventional spinning methods [44, 71, 72].
In addition, electrospinning is the other most common spinning method for fabricating collagen fibers with fascinating properties [72, 73] in which the collagen solution is gradually fed into the capillary spinneret held there by its surface tension force. When it encounters the

electric field, an electric charge (mostly positive) is induced on the fluid surface. Once the intensity of the electric field reaches a critical threshold, the repulsive electrical force dominates the surface tension force [48, 74-76]. Eventually, a jet of collagen chains is initiated to eject from the tip of the spinneret towards the minimum of the dipole energy (the grounded collector).

Both above approaches have been efficiently applied to collagen, from a mass of wet spun fibers with aligned orientation [27, 70] (Figure 3 and 5) to electrospun nanofibers with large surface area-to-volume (Figure 6) [3, 23, 29, 77].

As an example of wet-spinning, Stoessel et al. [27] characterized wet-spun continuous filament fabrication of gelatin as a derivative polymer of collagen with customized pre-treatment using a ternary raw materials of isopropanol, water and plasticizer, whereby superior tensile modulus (up to about 4 GPa) was achieved depending on the spinning set-up and the content of cross-linker/ plasticizer (triethylene glycol and ethylene glycol) using up to 200 wt. % of gelatin initial weight. The resulted fibers displayed wet stability depending on the solution contents and post treatments with cross-linkers and heating. This is an example that emphasize on value-added collagen chains by supportive materials to reach mechanical strength and amphiphilic properties. In fact, this complex procedure has been suggested for the design of textile structural design appropriate for clinical consumables. However, boiling point, latent heat of vaporization and toxicity of the highly used volatile solvents are important for wet spinning method to be considered.

By contrast, fiber formation using the electrospinning methods following the principals of electrohydrodynamics has received a great deal of attention due to the high flexibility of this processing method in controlling the fiber diameter, morphology, orientation, dimensions, and porosity, which do not necessarily involve coagulation chemistry or high temperatures to fabricate collagen fibers from solution[78]. More specifically, electrospinning as an inexpensive processing method has been applied to form collagen fibrous webs mimicing the native tissue architecture.

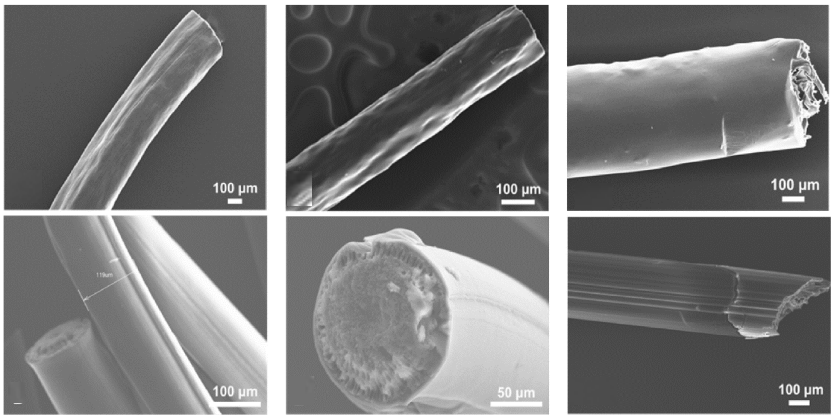


Figure 5 SEM images of wet-spun fibers from collagen-based materials (Gelatin and hydrolyzed fish collagen) obtained with varied solvents (i.e. 2,2,2-trifluoroethanol and dimethyl sulfoxide) and non-solvent coagulating medium conditions (i.e. acetone and ethanol) resulting in different fiber diameter in micron size (approximately from 110–450 µm). Reprinted with permission from (Ref. no. [68]) Copyright (2015) from Elsevier.

More specifically, since the native structure of collagen is mostly found as fibrous network/filament/fibril, the development of electrospun collagen can represent unique characteristics in nanoscale. For instance, Matthews et al. attempted to control the deposition of the electrospun fibers on a grounded collector to simulate the 3D geometric placement of collagen fibrils in native tissues from aligned to random deposition, Figure 6 [23]. Due to the

excellent inherent properties of collagen nanofibers, they believed that the electrospun nanofibers represent an ideal tissue engineering scaffold. They claimed that the collagen required to be selectively deposited to mimic the native tissue. Even though they observed a significant non-uniformity on the size of electrospun fibers, they suggested that the final shape adjustment of the accumulated electrospun fibers can be simply accomplished by electrospinning. Also, they concluded that both the type and source of collagen have a direct impact on fiber morphology as concluded by the electrospinning process of isotype collagen (type III vs. type I) from the source (type I placental vs. type I calfskin) [23].

Despite being appropriate to random deposition of collagen fiber [79], Figure 6A or along a defined axis [80], Figure 6B, the individually electrospinning of collagen fibers represent limited possibilities with regard to morphology protection in functional environments, structure customization and three-dimensional geometry [48, 81].

However, to achieve tailored physical properties, purified collagen can be blended, conjugated and grafted with/from some specific (bio)polymers and nanofillers before electrospinning. In addition, like conventional fiber spinning methods, collagen fibers from electrospinning are generally cross-linked to protect the morphology of fibers in high humidity conditions [45, 82, 83].

A B

Figure 6 A) SEM image of random orientation of electrospun type I collagen from human placenta, fiber diameter ranges from 100 to 730 nm. B) SEM image of electrospun collagen type I calf skin collected onto a rotating drum at 4500 rpm. Reprinted (adapted) with permission from (Ref no. [23]). Copyright (2002) American Chemical Society.

It is well known that electrospinning has the possibility to form customized fibers such as coaxial fibers using a coaxial spinneret. The process set-up is relatively simple that can be used for structurally and electrically incompatible polymers to avoid shear rates, high temperatures and surfactants, through in situ blending, or encapsulation while spinning the fibers[84]. In fact, a coaxial spinneret allows at least two different polymer solutions to be processed simultaneously in order to form a single coaxial fiber where the core contents are typically encapsulated by the shell [85-87]. This method provides better spinnability of collagen along with problematic polymers e.g. with low molecular weights, custom-made molecular conformation, and compositions with limited solubility [88, 89].

Zhang et al. [87] investigated the uniformity of collagen as the shell of PCL fibers, prepared by coaxial electrospinning, in comparison with rough collagen deposition by soaking; whereas Huang et al. [90] reported approximately the same procedure, while focusing on the fiber formation and mechanical characterizations of the core-shell fibers. Thus, they added a new approach to coaxial electrospinning of collagen that can be regarded as an advantage over conventional spinning methods, not only in developing functionalized fibers but also in elevating their mechanical properties. However, techniques such as coating or surface functionalization has limited collagen presence on the shell of fibers that are susceptible to

leaching by normal washing [61]. Hence, the spun fibers are subjected to crosslinking at finishing stage of process.

however, little research has been conducted on the natural based graft copolymers that are coelectrospun with sypolymers to increase the mechanical properties.

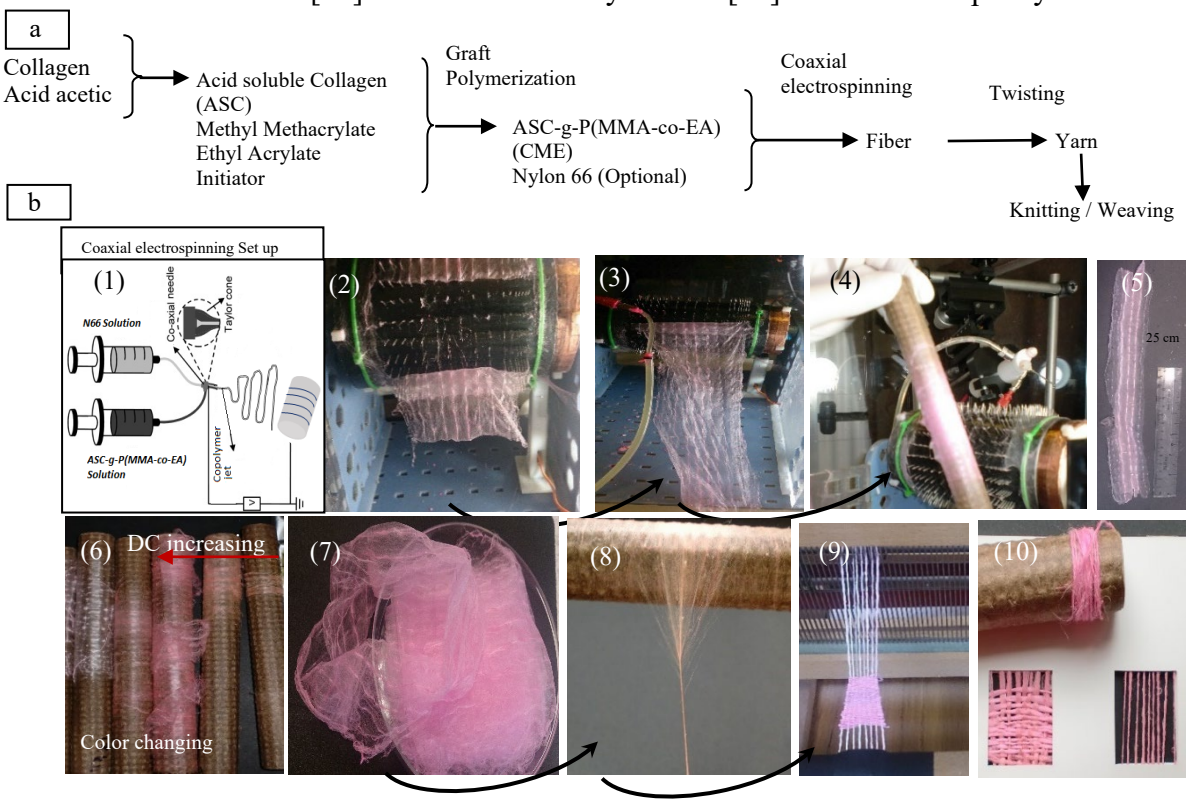
By contrast, we recently examined the spinnability of collagen with more problematic polymers containing two structurally and electrically incompatibles of collagen graft copolymers and poly amides [47]. In this work, collagen-g-poly (methyl methacrylate-co-ethyl acrylate) (CME) was used in the core components to take advantages of high density of chain entanglements and amphiphilicity of modified/branched collagen chains (CME), while Nylon 66 was as a reinforcing agent in the core of fibers; this spinning set up was performed free from any post treatment (cross linking). Custom-built electrostatics and supplementary bonding e.g. hydrogen bonds were identified as major factors for the design of reinforced CME/nylon 66 core-shell fibers, Figure 7 [47]. This approach towards collagen-based materials can simply expand the diversity of hydrophilic fibrous assemblies and their ever-increasing demand for multi-functional properties from collagen-based materials in a variety of applications.

However, single fiber formation of more than one component is not only influenced by the solution, process and environmental parameters, but it can be also affected by a new factor of solution-solution interactions in coaxial electrospinning. For instance, the inner and outer solvents can be a factor determining the immiscibility of the core and the shell components during fiber spinning [81]. Thus far, a number of researches have ascribed the core-shell fiber formation using a miscible solvent or the same solvent, for the core and shell components [85, 87, 91], even though the effect of miscible solvents has not been clearly explained.

Figure 7 (a) Process chart of CME/N66 yarn and potentially fabric production. (b) Coaxial electrospinning process and yarn twisting. (1) Coaxial electrospinning using rotating drum as collector. (2-5) Manually CME/N66 filaments were taken up around the nail drum (6) the color changing from pinkish yellow to yellowish pink by increasing the applied voltage that indicates the varied fiber in component. (7-10) The fibers were twisted into a yarn with clockwise twisting(S-twists) that is mechanically strong to be knitted /woven [47].

Viscosity and surface tension are critical factors for solutions to achieve a single fiber from both the core and shell components. This is due to the fact that the degree of polymer chain entanglements determines the viscosity of the solutions and intermolecular interactions cause the surface of the fluid to minimize the surface area. More specifically, high molecular weight polymers or increased polymer concentration lead to the formation of clogs/beads formation in the polymer jet, not allowing core-shell fibers to be stretched [92]. For instance, Zhang et al. [93] considered the effect of concentration on fiber morphology; they realized that, by increasing the core and the shell concentrations, the resulting fibers are of higher diameter. According to Lu et al. [81], the spinnability of the shell components is the most important factor in coaxial electrospinning. And, in our previous work, we also reported that by increasing the intensity of the electric field, different fiber content can be achieved from CME and Nylon 66[47]. These two examples suggest that the solutions are charged independently when encounter an external electric field, even though they are employed in the same process conditions and that the prominent driving liquid is the one with the larger electrical conductivity.

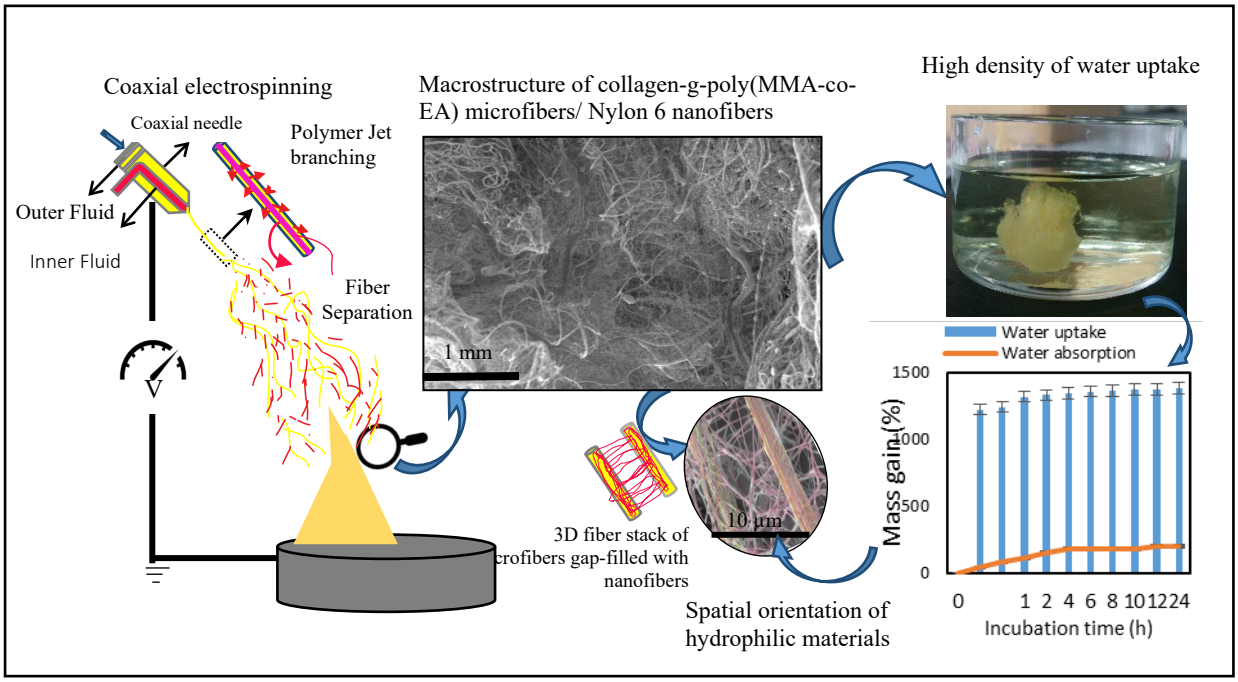
Hence, for each spinnable polymer solution, the solution conductivity must be in a range that often referred to as a "Leaky Dielectric" which develop electrohydrodynamics referring the deformation/motion of droplets by an electric field [94]. The leaky dielectrics and their responses to electric fields are discussed in detail in the references by Hohman et al. [74, 75], as well as schnitzer et al. [95] and earlier work by Saville [94]. The varied capacity of built-in



dipoles in leaky dielectrics appears in the shape of a different dimension of polarization; it can be developed that the surface charge density term can be formed depending upon the scaling of relevant orientation and dimension of build in dipoles. This can emerge a jump in voltage across the liquid-liquid interface between the two leaky dielectric media being placed in the same intensity of the electric fields; the varied chain orientation arises as a natural consequence of the interfacial boundary conditions for the dipoles forming a bimodal fiber formation of the core and the shell spinnable solutions, and that can be significantly detected

under certain electrospinning conditions,. We used this bimodal fiber formation of CME/Nylon 6 to fabricate a three dimension fiber stack, Figure 8 [48].
Our observations in the later work also revealed the structure of the varied chain orientation at the liquid-liquid interface (Figure 8)[48], which shows how interfacial chain orientation may arise under strong imposed electric fields. This also submits that the liquid polymer jets of each spinnable polymer solution in the core and the shell can be drifted apart under the same imposed electric field depending on the varied velocity of each, which is appeared to a leading order forming interfacial interactions of collagen-based macrostructure in three dimensions. Hence, to fabricate tailored uniform coaxial fibers, the optimal solution-solution parameters require to be determined [47, 48].
In addition, to produce higher volume of fiber spinning from collagen-based materials either as mixed-fibers or multi-layered fibers, multiple spinnerets are utilized sequentially or concurrently. This allows a set of polymeric solutions to produce higher volume of nanofiber nonwoven mat with desired mechanical, chemical and biological properties. For instance, Kidoaki et al. [96] designed ordered mesoscopic assemblies of scaffolds and matrices of nano to micron-sized fibers for tissue-engineering devices comprising multi-layering and mixing electrospinning. They used four components of type I collagen, styrenated gelatin (ST-gel), polyurethane (PU), and poly (ethylene oxide) (PEO). A tri-layered mesh was electrospun sequentially by (type I collagen, ST-gel, and PU). The mixed fiber mesh of PU and PEO was also formed by simultaneous electrospinning. This multi-layering method was useful to control the eventual composition and mechanical properties of the fibrous matrices. This method is particularly suitable for composites with a multilayer structure, such as protecting clothes, filtration materials, and tissue engineering.

Several new spinning methods have been also developed to produce fibers. Infusion gyration method is one of them, which is in its early stages; in this innovative method, simultaneous solution feeding and centrifugal spinning was used to fabricate micro- to nano-fiber in large scale [97, 98]; according to Hong et al. [99] and Heseltine et al. [100], tailored fibers are achieved by controlling the process and solution/melt parameters such as polymer concentration, molecular weight, viscosity and rheology, infusion/flow rate, working pressure, and rotational speed. Even though, to the best of our knowledge, there is no collagen-based fibers have been reported via this technology, but it seems PEO with high molecular weight (above ~200,000 $g.mol^{-1}$), is the most studied polymer through this method and potentially can be used for mass production. From our understanding, this methodology is relatively similar to dry spinning when high chain entanglements from concentration and viscosity is crucial for fiber spinning, and in order to extruding, drawing and stretching the fabricated fibers; instead of the gravity and mechanical force, centrifugal force is used to



produce fine fibers. This easy to adjust method seems to be used when the spinnable collagen-based solution meets the criteria of the conventional spinning methods as will be discussed later.

Figure 8 A block diagram of One-Step Fabrication of Three-Dimensional Fibrous Collagen-Based Macrostructure with High Water Uptake Capability by Coaxial Electrospinning [48].

Apart from the processing methods, collagen fibers can be produced into micro size ranges from 89 to 400 µm [67, 68] and also into nano size range from 50 to 1200 nm by electrospinning [29, 77, 101]. For instance, Meyer et al. [67] used cylindrical and conical nozzles with diameters between 250 and 500 µm to yield fibers in the range of 89–170 µm by wet spinning whereas, Shih et al. [101] achieved three ranges of fiber diameters (50–200, 200–500, and 500–1,000 nm) from concentrations of 4, 8 and 12% w/v, respectively. These findings prove that collagen has the capacity to be spun in various fiber diameters from nano– to micron scale.

In comparison to other processing techniques, conventional spinning methods have delivered highly aligned fibers in high quantity, and electrospun collagen nanofibers can be formed in multiple geometries such as randomly or along a defined axis, and dramatically increases the

surface area-to-volume ratio, tunable diameter, and porosity. While, the important challenges of collagen fiber spinning via conventional methods may be large amounts of organic solvents employed during the process, time-consuming production and complexity of the process set-up, which can make it unsuitable for practical use in small scale; however, the fibers produced via conventional spinning methods generally exhibit an enhanced mechanical strength due to the post drawing of the spun fibers in comparison with electrospun nanofibers [27, 42], the fiber taking-off/ detaching from the collectors in electrospinning is still in its early stages of research, hence the dimension of accumulated nanofibers is not exceeded the surface of collector. On the other hand, electrospun collagen fibers has shown fascinating properties that have not been reported via other spinning methods so far; such as adjustable encapsulations, in-situ blending with incompatible materials and single- and multi-layered composites. This has turned the electrospun collagen fibers into the highly controllable multifunctional fibrous assemblies. The weakness of electrospinning methods in collecting the spun fibers when mass production is tailored, may be answered in future using similar approaches implemented in conventional spinning methods such as transfer cylinders /rollers over decades of research.

1.3 Effect of collagen sources spinnability of purified collagen chains

As previously mentioned, collagen as a suitable biopolymer for fiber-spinning can be extracted and isolated from both mammalian and marine tissues[102]. The most studied collagen is type I from bovine /calf skin [68, 82]. Some other sources of collagen have been also studied; for instance, Choi et al. [83] examined a fish collagen-based composite fiber to support cell biological activities and also a similar electrospinning investigation into collagen from a cold water fish has been reported by Hofman et al. [103]. They electrospun fish collagen from multiple molecular conformations (native triple helical chains, denatured whole collagen chains, and denatured gelatin) and suggested that the varied source of collagen presenting different molecular weights. And, molecular weight is an important factor determining the morphology and quality of the electrospun fibers where low molecular weight gelatin failed to form fibers. They also found that very high collagen concentrations (above 20%) are essential to fabricate the electrospun fibers.

In a similar approach, the source of collagen has received attention as a factor that can affect the morphology of fibers, according to Tronci et al. [68]. They evaluated wet-spun fiber formation using collagen with varied molecular weight; hydrolyzed fish collagen and gelatin from bovine skin having low and high molecular weights, respectively. They found that the morphology and diameter of the wet-spun fibers are significantly affected by the molecular weight. As mentioned earlier, Matthews et al. [23] also explained the electrospinning of collagen type I from two different sources; human placenta and calfskin. Collagen type I from human placenta resulted in less uniformity in fibers with larger diameters. Furthermore, Zeugolis et al. [104] electrospun a set of collagen solutions prepared from five different samples of varied batches of type I bovine dermal atelocollagen. They observed that even in spinnable solutions, the fiber morphologies varied from one to another. They speculated that this variation is due to differences in amino acid content related to the age and even the race of the source.

To the best of our knowledge, apart from the varied morphologies that may occur depending on the collagen source and type; the material and biological properties of spun fibers based on the type and source of collagen has received less attention to be considered. With the assumption that the type and source of collagen has no effect on functionality of the collagen spun fibers, however they can be affected by the size of the fibers affecting from varied molecular weight of collagen chains and can be also counted as an important factor determining the fiber spinnability and properties; varied molecular weights is not only from

the collagen source but also within the same source; simply affected by age, race, and injuries. This factor can cause considerable challenges for the reproducibility of the pure collagen fibers for specific applications, and this can be the main reason for using gelatin with a pre-identified molecular weight as a collagen derivate product in several reports of successful spinning of gelatin preparations.

1.4 Effect of solvents on spinnability of purified collagen chains

Solvent selection is critical for collagen spinning. Typically, organic solvents, such as 1,1,1,3,3,3-hexafluoro-2-propanol (HFP), 2,2,2-trifluoroethanol (TFE), or acids (tri-fluoro acetic acid (TFA), acetic acid, hydrochloric acid) have been extensively used to dissolve collagen to prepare spinning solutions. Among these denaturing solvents, HFP, TFE, and TFA with low boiling points have been used as common solvents in electrospinning. HFP and TFE lead to the loss of the triple helical conformation of collagen due to the damage of the delicate structure of collagen [29], even though the high concentration of collagen can partially hinder the drawbacks of these strong solvents. This has adversely affected the fiber formation of pure collagen through wet spinning and electrospinning. Tronci et al. [68] and Hofman et al. [103] reached to this conclusion to apply high concentrations of above 20% to obtain spinnable solutions to be spun through wet spinning and electrospinning, respectively.

Even though it seems that the denaturation of dissolved collagen before and during the process is a function of the proton donor or acceptor ability of the solvents (the concentration of H+), and the availability of proton-acceptor sites of the solute (collagen) in proton-donor solvents and vice versa, there is little information about the formation of solute-solvent hydrogen bonds/repulsion and their associated effect on the collagen denaturation. However, the effect of solvents became more critical when Zeugolis et al. [105] claimed that electrospinning of collagen from HFP leads to the formation of readily water-soluble gelatin fibers, due to seriously denaturing the uncoiled structure of the collagen chains and there has been mention that perhaps electrospinning collagen is simply an expensive way to produce gelatin. Even though, their findings were also examined by other research groups [29, 106, 107], there are some other research groups that believe collagen native structure perfectly preserves in specific collagen solutions with high concentration through electrospinning [107].

However, there is little information available about the probable effects of external electrostatic field on repulsive forces onto delicate structure of collagen that might deteriorate the impact of solvents. On the other hand, to the best of our knowledge, there is no comparison between spinning methods using the same solution parameters to evaluate the effect of process parameters on denaturation of the collagen chains.

However, the impact of solvents has been examined in both conventional and elecrtspinning methods separately [67, 107]. For instance, according to Liu et al. [106], the collagen nanofibers from acetic acid (as a weak acid) showed more collagen preservation in contrast to the collagen fibers from HFP. And, in a separate study, it was suggested that in collagen spinning, applying weak acids such as acetic acid can maintain a larger segment of the structure of collagen chains. This result was explained further by Qi et al. [107]; they claimed that the concentration of H^+ in the solvent plays a key role in the dissolving rate of collagen when pH = -log (H^+). It is well-defined that collagen chains are amphoteric polyelectrolyte identified by carboxyl groups (–COOH) and amino groups (–NH2). Since the hydrogen ions of acidic solvents are simply ionized in collagen solutions, hydrogen ions (protons) are partly adsorbed onto the surface of collagen chains and the rest can be freely moving within the solvent: $R–NH_2 + H^+ \rightarrow R–NH_3^+$

where R– is the rest of the collagen chain, and $–NH_2$ is the adsorption site for hydrogen ions available on the surface of the collagen chains [107]. When more free hydrogen ions are

produced by a stronger solvent, a higher amount of hydrogen ions is attracted onto the surface, which increases the electrostatic repulsion force between the collagen chains. In the same study, Qi et al. also realised that collagen chains can be significantly degraded with highly increased H^+ concentration in a spinning solution.
In brief, they suggested that when pH of a collagen spinning solution is lowered to ≤ 3.0, the high availability of H+ causes collagen chains to permanently unwind into random-sized poly-peptide chains, resulting in partial failure of the biological activity of collagen chains. On the other hand, the possibility of replacing fluoro-alcohols and HFP with a mild solvent, has been examined in various studies for collagen fiber spinning. For instance, to preserve the characteristics of collagen chains, a mixture of water, HCl, and DMSO at pH of about 4.0 was used to wet spin collagen fibers by Meyer et al. [67] and Elamparithi et al. [108] used a mixed solvent of acetic acid and DMSO in a ratio of 93:7. In a similar work, Tenchurin et al. [109] realized that for efficient manufacturing of fibrous collagen-based materials by electrospinning, the search on optimal rheological parameters is of the great importance. It was revealed that optimal parameters for electrospinning of highly concentrated collagen dispersions can be achieved by adjusting of the concentration of acetic acid, temperature (up to ~30oC), and stirring speed. Finally, Qi et al. [107] concluded that a 57 % of native collagen chains can be preserved by using a benign sodium acetate/acetic acid buffer solution at pH 3.0. Furthermore, Sizeland et al. [102] previously suggested that by selecting a proper solvent within benign solvents, the architecture of electrospun collagen nanofibers can be compared with that of native collagen fibrils even from a source with low molecular weight such as Hoki (Macruronus novaezelandiae) skin. This example interestingly implies that fiber spinning results in the reformation of collagen fibers with similar nanostructural properties to those found in native collagen tissues, even though from their studies they concluded that the lack of internal fibril structure exists, in contrast to the hierarchical structure of native collagen that provides mechanical stability, strength, and elasticity for native tissues [10, 12].

1.5 Effect of additives/reinforcements on the processability of collagen chains

As previously mentioned, collagen fibers may lose its physiochemical properties from the hierarchical structure passing through two stages: (1) in isolation and extraction and (ii) in processing [57, 102]. Therefore, collagen fibers basically represent poor mechanical properties, and thermal and water instability. Accordingly, polymer/nanofiller additives have been found to be important in improving the physiochemical properties of collagen fibers [56]. Typically, the presence of additives in the collagen-based polymer matrix serves to reinforce the mechanical behavior of the composite nanofibers.
A variety of additives have been proposed for this purpose including compatible synthetic polymers, clays, synthetic silicate nanoparticles, hydroxyapatite and carbon nanofillers [110, 111]. PCL, poly(3-hydroxybutyrate-co-3-hydroxy valerate) and Poly(glycolic acid) (PGA) are examples of synthetic polymers that have been incorporated with collagen fibers as a reinforcing agent for tissue engineering [58, 87].
In general, composite fibers can be prepared mostly through three processes including blending [43], coaxial spinning [90], and fiber mixing and multi-layering [96]. As discussed earlier, apart from the spinning methods, the compatibility of the solution components is a critical aspect in polymeric blends to fabricate the composite fibers. Coaxial spinning is another method in common between conventional spinning methods and electrospinning methods. However, coaxial electrospinning is more straightforward to produce composite electrospun fibers in which collagen can be electrospun as either the core or the shell layer [85, 87]. Due to its hydrophilic behavior, collagen can be processed as the shell with a synthetic polymer in the core as reinforcement agent [47]. Again, solution-solution

interaction of the components in the core and the shell encountering an external electric field have been considered in producing the coaxial collagen nanofibers [81, 87, 112].

To produce multi-layered collagen-based composite fiber mesh, different polymeric solutions is spun sequentially which is only possible via multi-layering electrospinning [11]. Hence, each layer of the composite fiber mesh has its own structural and physical features. For instance, Baek et al. [11] suggested an interesting fibrous multilayered composite comprising cell seeded on/ encapsulated into an aligned electrospun collagen type I scaffolds which was subjected to examine tensile mechanical properties. Apart from multi-layer electrospinning, simultaneously mixing and electrospinning comprises simultaneous electrospinning of at least two different polymeric solutions from separately fitted spinnerets to produce composite collagen fiber mesh. For instance, a double-spinneret electrospinning technique prepared with multilayer 3D scaffolds stacking PLGA microfiber membranes alternately to micro- /nano-mixed fibrous membranes of PLGA and collagen. By controlling the density of collagen fibers in multilayered scaffolds, the adhesion, proliferation, and osteogenic differentiation of cells was investigated, while homogeneously dispersion of hydroxyapatite nano rods (nHA) in the collagen solution improved the osteogenic properties of the fabricated multilayer scaffold. Again, this strategy is like the multi-layering method, only can be performed through electrospinning processing methods.

To produce composite collagen fibers, carbon nanofillers are a relatively new class of fillers that have been recently subjected to a wide variety of applications due to their high specific surface [111, 113-116]. In the last few years, one-dimensional carbon nanotubes or nanowires (CNTs), and two-dimensional nano-sheets (graphene and its derivatives) have been explored extensively [117]. According to Chi and Wang [118], incorporated functionalized CNT in collagen can generate biocomposite fibers by electrospinning. Considering fiber dimension, alignment, mechanical strength, electrical conductivity and biocompatibility, the addition of CNT reinforced the strength of the scaffolds and rendered the fibers electrical conductivity to not only facilitate the fiber formation but also grant the fibers an additional functionality that can be utilized for cell stimulation.

In general, Nanofillers is normally introduced to a collagen component during solution preparation. The solvent preperation refers to a suspension in which a nanofiller is dispersed, then simply added to a solvent where collagen is dissolved as a host polymer [63, 111]. This method is classified as a modification based on non-covalent conjugation [119-121]. In the last few years, several research groups have attempted to apply this methodology to enhance the mechanical properties of spun fibers including collagen and other biopolymers [63, 122, 123].

Thus far, the principle method for formulating nanofiller/collagen composites has been processed by the electrospinning methods, which among the spinning methods has received extensive attention from several research groups [62, 111, 119, 122, 124, 125]. This method can further provide the possibility for electrospun collagen fibers to be physically cross-linked and mechanically reinforced by the presence of nanofillers in collagen composite fibers [63, 123, 125]. However, among the conventional there are some interesting work focusing on aligning the collagen fibers through gel spinning [126]. According to Green et al 2017 [126], this conventional method can result in a manually formed bundle of collagen/nano-carbon fibril-like fibers. They characterizeed the molecular and fibrillar alignment, where D-banding was observed in the spun fibers – consistent with native collagen. The results of this example show the multi-scale influences of a rigid inclusion on molecular ordering and fibril alignment

Affecting the tensile strength of collagen fibers in both wet and dry states that could be comparable to the native materials.

Apart from the spinning method, for the composite collagen/nano filler fibers, the morphology and dispersion quality of the nanofillers within the composite fibers has been found to play a significant role in influencing collagen molecular orientation and fiber alignment. In other words, the non-uniform dispersion of the nanofibers within the spinning solution may be a significant challenge that can considerably affect the processability, as well as the morphology and mechanical properties of composite nanofibers; however, this challenge has been frequently reported within any nanofiller/polymer composite that resolved by using functionalization methods when low concentration of nanofillers is found to be more effective during fiber spinning process [62, 63, 114, 122, 123, 127].

Furthermore, in-situ polymerization is another approach that can be used to prepare uniformity in the dispersion of nanofillers within the medium, benefitting from stronger interactions of both covalent and non-covalent bonds between the nanofiller and host polymers [116, 128]. This method can be employed for nanofillers with active sides such as graphene oxide (GO) [111, 119], which is a compromise of graft and conjugation modification methods on collagen chains. This approach was applied in our research due to the relatively low performance and the efficiency of the grafting onto collagen chains that was significantly enhanced with the presence of GO [56]. In situ polymerization represents mixing some nanofillers to the collagen in a good solvent with the presence of initiator, followed by addition of complementary optional monomers. The nano fillers due to their large accessible surface area for initiated collagen chains allowed for an accelerated polymerization with highly improved grafting performance and efficiency. This can be another method to prepare homogeneous nanofiller/ collagen composites to examine the processability of the achieved nanocomposite through electrospinning processing methods. The collagen/GO graft nanocomposite fibers were found with sufficient stability without need to post treatments such as cross-linking. However, due to the size of the nanofillers and the random geometrical placement of the nanofiller within the fibers, the uniform morphology of the electrospun fibers was observed in low addition of GO loadings (< ~0.5 wt%). It was also found that humidity and temperature play key roles in the degradation rate of collagen/GO graft nanocomposite fibers; above 50 ^{o}C, GO is not as stable as branches on the surface of collagen chains, which signifies varied strength of the intrer and intra molecular interaction of the composite when the polymers reach their glass transition temperature (Tg) [56, 57].

In general, nanofillers have been extensively used as a reinforcing agent that can simply improve the functionality of the collagen-based nanocomposites by controlling the mechanical properties and degradation rate of collagen, however there are some factors in nanofiller selection such as the aspect ratio, geometry such as (i) nanosheets or layered flakes (e.g. clay, graphene), regualar/irregular spherical(e.g. silica, metallic nanoparticles), nano rods (carbon naotubes, celloluse nano crystals), concentration/loadings, active end groups that determine the efficiency of the nanofillers within the fibers [47, 48, 51, 56, 57]. In other words, to obviate any chemical modification onto the collagen chains, it seems that the combination of strong chemical moieties on nanofillers with biologically active collagen chains can be a key to some of the future's innovative research. This can be a powerful strategy for the selective conjugation of recombinant collagen/nanofillers domains with peptides/polymers through a host-guest chemistry approach as an outlook on collagen fiber reformation mimicking the structural molecular arrangements of native collagen fibers in tissues.

During the last two decades, the challenges associated with all spinning methods for collagen fibers consisting of fiber discontinuity, low fiber alignment, inability to achieve sufficient strength, reproducibility and failure to replicate the internal fibrillar structure consistent with native collagen that due to the lost properties from hierarchical structure of

collagen fibers in native tissues, has been reported. Due to the excellent biological properties of collagen chains, several researches have been performed to modify collagen chains; avoiding the denaturation of collagen chains during the extraction and process, and strengthening the collagen chains via additives such as natural and (semi)synthetic polymers, nano fillers and reinforcements via spinning methods. While the mechanical properties of wet- /dry- spun fibers are mostly from the post-treatment of the fibers e.g. drawing/stretching, the fiber taking-off/ detaching from the collectors in electrospinning is still in its early stages of research. In this review, the spinning methods applying for collagen fiber formation was summarized and their strategy to achieve the modified and reinforced collagen fiber was studied.

Conclusions

Collagen is an important biomimetic material. Several attempts to spin this biopolymer have proved difficult due to significant denaturation and degradation occurring while being dissolved in a solvent and spun. Purified collagen chains can be modified to add stability to their structure combating the negative impact of processing as post-treatment such as exposing to crosslinking agents, blending with other polymers and as pre-treatment such as graft polymerization when monomers are subjected to be grafted onto the collagen chains. While pre-treatment can change the viscosity of the spinning solution, they can significantly preserve the collagen chains in process.

The studies have shown how to process a composite fiber which can consist of collagen-based material with (in)compatible polymer leading to varied physical properties; thermal, mechanical and degradability affected by chain orientation and intermolecular interactions between polymer chains. The impact of this review is to show the ability of collagen to be processes via different spinning methods making it more stable via blending, crosslinking, grafting, and conjugated with new end groups. In consequence, the new developments of the collagen fiber reformation can generate new properties and possibilities of numerous medical and industrial end uses.

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